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Published in:
Current Opinion in Biotechnology

DOI:
[10.1016/j.copbio.2012.12.001](https://doi.org/10.1016/j.copbio.2012.12.001)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2013

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Steele, J., Broadbent, J., & Kok, J. (2013). Perspectives on the contribution of lactic acid bacteria to cheese flavor development. *Current Opinion in Biotechnology*, 24(2), 135-141.
<https://doi.org/10.1016/j.copbio.2012.12.001>

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Perspectives on the contribution of lactic acid bacteria to cheese flavor development

James Steele¹, Jeffery Broadbent² and Jan Kok^{3,4}

It has been known since the 1960s that lactic acid bacteria are essential for the development of cheese flavor. In the ensuing 50 years significant research has been directed at understanding the microbiology, genetics and biochemistry of this process. This review briefly covers the current status of cheese flavor development and then provides our vision for approaches which will enhance our understanding of this process. The long-term goal of this area of research is to enable technology (i.e. cultures and enzymes) that results in consistent rapid development of cheese variety-specific characteristic flavors.

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Current Opinion in Biotechnology 2013, 24:135–141

This review comes from a themed issue on **Food biotechnology**

Edited by **Elaine E Vaughan** and **Jeroen Hugenholtz**

For a complete overview see the [Issue](#) and the [Editorial](#)

Available online 29th December 2012

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<http://dx.doi.org/10.1016/j.copbio.2012.12.001>

Introduction

Lactic acid bacteria (LAB) dominate the microbiota of ripening cheese and include deliberately added strains (e.g. starters and adjunct cultures) and adventitious species (primarily nonstarter LAB or NSLAB). Directly after manufacture the starter culture frequently exceeds 10^9 colony-forming units (cfu) per gram of cheese. The harsh cheese-ripening environment (no residual lactose, pH 5.0–5.3, 4–6% salt in moisture, 5–13°C) results in a gradual decline in starter viability. Some of the dying starter cells undergo autolysis, which releases intracellular enzymes and cellular components (e.g. sugars and nucleic acids) into the cheese matrix [1]. At the same time, NSLAB populations (initial numbers typically less than 10^2 cfu/g) begin to increase and plateau at cell densities of 10^7 – 10^8 cfu/g after 3–9 months of aging [2]. NSLAB populations in Cheddar cheeses may be quite diverse,

but are usually dominated by facultatively heterofermentative lactobacilli [2,3].

Cheese flavor development is a dynamic biochemical process that is impacted by: first, the type and composition of milk; second, processing conditions; and third, the microorganisms and enzymes present in the cheese matrix (Figure 1). The cheese microbiota is the primary source of enzymes influencing flavor development. The substrates for these processes are carbohydrates (glycolysis), organic acids (i.e. citrate), lipids (lipolysis), and proteins (proteolysis). Of these biochemical processes, proteolysis and the subsequent amino acid (aa) catabolism are of universal importance in flavor development, regardless of the cheese variety. Therefore, this review will focus on these two processes and the related process of bacterial autolysis (Figure 2). Readers interested in a more comprehensive review of cheese flavor development are directed toward the following reviews [4–6].

Primary enzymes and pathways

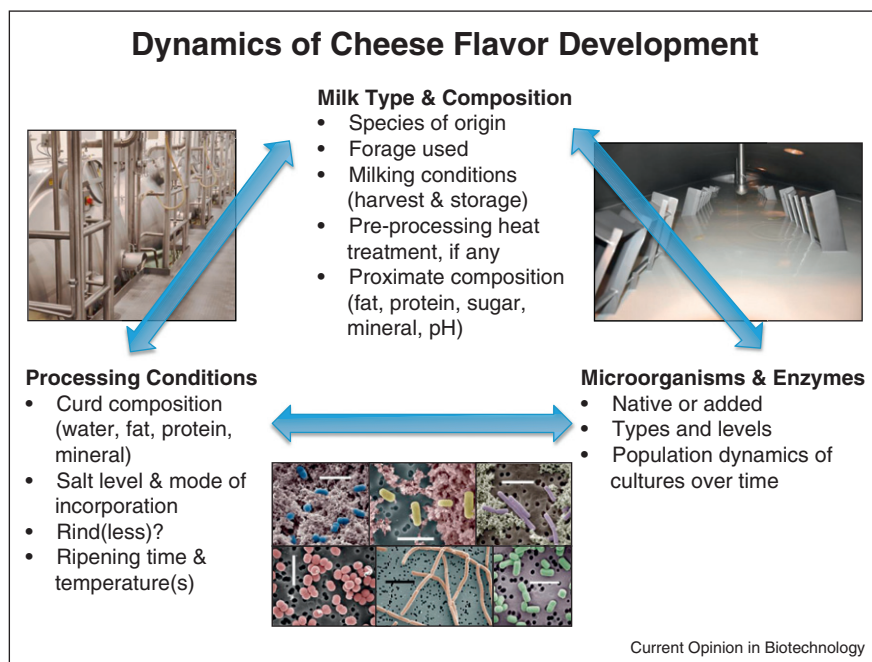
Protein and peptide breakdown

The first step in flavor development from milk proteins by LAB is the hydrolysis of casein by a cell envelope proteinase of which several types have been identified [7]. *Lactococcus lactis* PrtP, the best-characterized LAB proteinase to date, is initially produced as a pre-pro-enzyme. Through a mechanism not yet understood, the PrtM chaperone induces PrtP to activate itself by cleaving off the pro-sequence, after which mature PrtP is secreted and covalently anchored to the bacterial cell wall by a sortase [7]. *L. lactis* PrtP has a rather broad specificity and is capable of producing more than 100 different oligopeptides from caseins. The oligopeptides are internalized by Opp and subsequently hydrolyzed by a host of intracellular peptidases [7]. The exact composition of the peptidase complement will determine the ultimate breakdown profile of the casein-derived peptides and, thus, which peptides and aa will contribute to flavor. However, only minor flavor differences have been observed in cheese trials conducted with LAB with altered peptidase activities, although significant changes in the aa pool were observed, indicating that the conversion of aa to flavor compounds is the rate limiting step in flavor formation from proteins [7].

Amino acid catabolism

Much of the research on aa catabolism by LAB has been directed toward the fates of aromatic, sulfur-containing, and branched-chain aa because of their key roles in aroma

Figure 1



Dynamic nature of cheese flavor development.

development. Two major pathways convert aa to flavor compounds: elimination reactions catalyzed by aa lyases and pathways initiated by aa aminotransferases (see Figure 2). The aa lyase reaction of greatest interest in cheese flavor formation is the production of methanethiol from methionine. This reaction is carried out by two distinct C-S lyases, cystathionine β -lyase and cystathionine γ -lyase, which are present in most LAB [8]. Two aminotransferases, aromatic and branched-chain aminotransferases, have overlapping specificities and convert methionine, aromatic, and branched-chain aa to their corresponding α -keto acids [5]. The α -keto acids are key intermediates in aroma development and can be subsequently converted to α -hydroxyacids, acetyl-CoA derivatives and aldehydes, respectively (Figure 2). The aldehydes may be subsequently converted to alcohols and carboxylic acids. The wide variety of aroma compounds these pathways produce are critical to both beneficial, as well as detrimental flavor development in cheese [4]. Understanding the factors that determine the fluxes through these competing pathways will be essential to consistently and rapidly deliver cheese variety-specific flavors.

Role of starter cell lysis in flavor enhancement

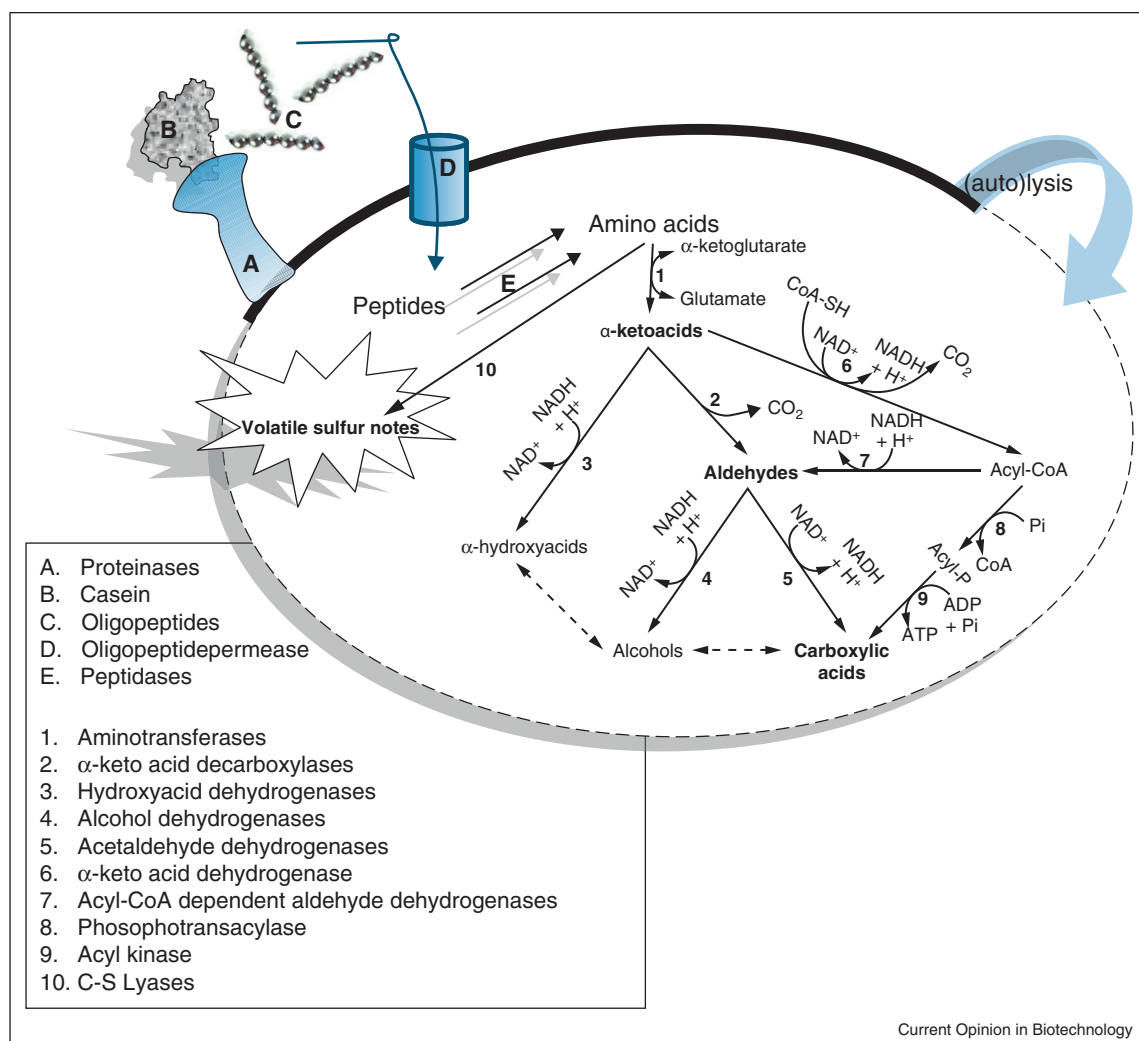
Lysis of starter bacteria can affect cheese flavor development through release of intracellular enzymes such as peptidases [1], and molecules that influence NSLAB growth. Lysis can occur via the activity of cell wall-degrading enzymes of the cells or through bacteriophages, either virulent or through induction of prophage [1]. Various

methods have been employed to increase the rate of starter culture lysis (e.g. by constructing or selecting for fast-lysing strains), using bacteriocin producers as adjunct cultures, or employing bacteriophage lysins either alone or in combination with the corresponding holin [1]. Overexpression of peptidoglycan hydrolases of *L. lactis*, as well as two phage-derived lysins increased lysis and release of intracellular peptidases [9,10]. Overexpression of the autolysins AcmA/AcmD or the phage r1t lysin LytR led to increased cellular lysis in cheese, resulting in a slight improvement in overall sensory quality of the cheese but not to changes in bitterness [10]. Strains showing a high degree of lysis are considered to be superior at flavor development and have been included in commercial products [[11]; E. Johansen, personal communication].

Post-genomics approaches to LAB cheese flavor enhancement

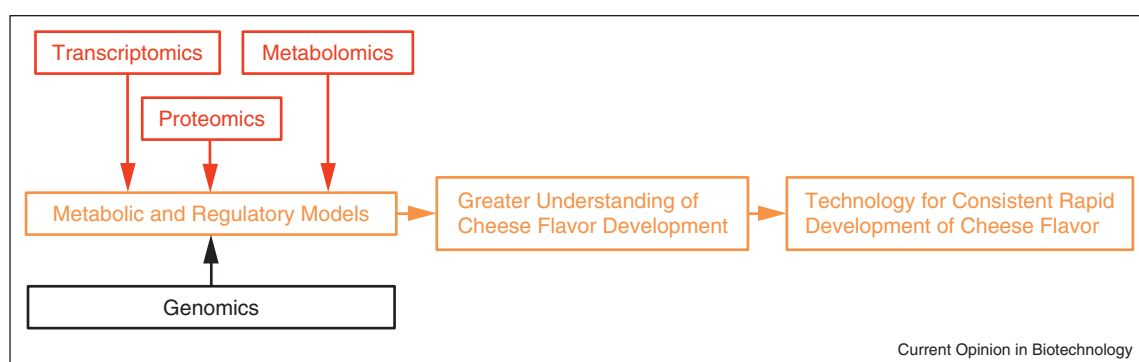
Systems biology, which involves the study of an organism as an integrated and interacting network of genes, proteins, and biochemical reactions, is an exceptionally powerful means to obtain detailed understanding of an organism's functions (for a recent review, see [12]). The foundation of this approach is the availability of genome sequences for the organisms of interest (Figure 3). To date, more than 140 draft (gapped) or finished LAB genome sequences are publicly available, representing more than 50 LAB species (excluding enterococci and pathogenic streptococci) [13]. Notably, genome sequences for other bacterial species involved in cheese flavor production are also available,

Figure 2



A generalized scheme of cheese flavor development.

Figure 3



The application of systems biology to cheese flavor development.

namely *Propionibacterium freudenreichii* subsp. *shermanii* [14] and *Brevibacterium linens* [13], which play roles in both appearance and flavor development of certain cheese types. Many fungi also make key contributions to cheese flavor [15], and work is needed to begin genome sequence analysis of these microorganisms.

Genome-based and microarray-based comparative genomic studies have already yielded remarkable advances in fundamental knowledge of LAB evolution, genetics, physiology, diversity and metabolism that are directly relevant to cheese technology [16–18]. For example, experimental evolution [19,20•] has recently confirmed that genome decay is a primary mechanism by which LAB have adapted to the milk environment and demonstrated that this approach can result in the isolation of plant isolates capable of rapid growth in milk. Importantly, this approach shows how industry could develop entirely new starter cultures. Comparative genomics has also provided a means to better understand and assure the ‘generally recognized as safe’ status of LAB in dairy foods [21–24], and most notably, revealed species-specific and strain-specific blueprints for the cellular networks and enzymes that contribute to flavor development in aged cheese [8,25,26,27••].

Transcriptomics

Whole-genome transcription analysis was first utilized in the genetic analysis of *L. lactis* [28]. Initially used to study the global effects of internal (mutations) or external (growth conditions) perturbations on the transcriptome, time-resolved (chrono-)transcriptomics has recently also been performed to follow effects in time. Thus, it has been possible to uncover and exploit the function of unknown genes [29], and describe the regulons of a number of important pleiotropic (global) regulators of among others, flavor formation (CcpA [30]; CodY [31]). Many stress conditions, several of which are encountered by LAB under cheese-making conditions, have been examined to identify key genes, pathways, and gene regulatory networks involved in these responses. The combination of transcriptome and proteome measurements has been especially revealing [32]. Most of these studies used laboratory media and the laboratory strains *L. lactis* ssp. *lactis* IL1403 or ssp. *cremoris* MG1363, sometimes endowed with plasmid(s) that enabled growth in milk-based medium. However, translating results from these studies to industrial conditions or to predict their effects on flavor formation remains a challenge. Recently, the transcriptomic responses of *L. lactis* were measured in (model) cheeses or under conditions designed to more or less mimic the cheese manufacturing process [33••,34••,35]. What is apparent, apart from the actual responses to, for example, carbon limitation, temperature, salt and acid stresses displayed by the specific strain(s) used in these studies, is that complex interactions take place between species and strains in mixed-strain cheese

starters. Future studies along the lines pioneered in [36] are needed to untangle the contribution of mixed-culture associations to the quality of fermented foods.

Proteomics

Proteomics, the study of the protein complement produced by an organism or a biological system [37], yields data that are more directly related to physiological state than transcriptomics data, as not all mRNAs are translated into proteins. However, the high protein content of the cheese matrix complicates proteomic analysis of LAB in cheese. Notwithstanding this, several proteomics studies have appeared detailing the protein/peptide content of milk and fermented milk products, or for example, the influence of lactation on proteolysis during cheese ripening [38,39]. Proteomics has also been used to describe the composition and/or functionality of the microbiota in cheese or under cheese production-mimicking conditions. Thorough reviews of the topic, also focusing on the pros and cons of the various technologies, are provided in [38,40]. The fact that starter culture strains are under stress during cheese production and make certain choices in their metabolism in response to the changing environment was clearly reflected by the differential identification of glycolytic enzymes and various bacterial stress proteins [41–43]. All in all, these studies have provided snap-shots of what might be going on in milk, the cheese matrix, or the SLAB and NSLAB acting during the fermentation process. The major next challenge is to apply quantitative proteomics, which would allow performing differential protein expression studies in a way similar to what is done in transcriptomics research [44].

Metabolomics

Metabolomics is the ‘systematic study of the unique chemical fingerprints that specific cellular processes leave behind’ [45]. This is a critical ‘omics’ for understanding cheese flavor development, as flavor results from the accumulation of LAB metabolites. Metabolomics has facilitated two major advances in understanding of cheese flavor; first, worldwide efforts to identify ‘aroma-active’ small molecules in ripening cheeses produced a ‘library’ of prospective flavor compounds [46]. This library subsequently empowered researchers to link specific molecules to desirable or undesirable cheese flavor attributes [47–51]. Recently, metabolomics has been used to explore NSLAB growth requirements [52•] and understand cellular flux in production of key flavor compounds [53••]. Moving forward, we anticipate metabolomics and systems biology approaches will reveal new strategies for optimization and acceleration of flavor production by bacteria and fungi in cheese.

Metabolic and gene regulatory models

Genome-scale metabolic and gene regulatory models link genomic data to biochemical reaction networks that define cellular processes [54,55]. These models can be

utilized to understand genotype–phenotype relationships and to compare different organisms. Additionally, they can provide the framework for the integration of transcriptomics, proteomic, and metabolomics data (Figure 3), thus offering a unique global view of cellular physiology and how microbial cells respond to environmental changes. Genome-scale metabolic models are now available for *L. lactis* [56], *Lb. casei* [Broadbent and Steele, unpublished data], *Lb. plantarum* [57], *Lactobacillus reuteri* [58], and *Streptococcus thermophilus* [59]. Of particular interest, the latter study compared the three published LAB metabolic models, the aa requirements of these three organisms, and their ability to produce volatile compounds [59]. The authors concluded that *S. thermophilus* LMG 18311's relatively low aa requirement and ability to produce a broad range of aa-derived volatile compounds, compared to the other two organisms, was the result of this strain's relatively complete set of aa biosynthetic and aa converting enzymes. This example shows just how important it is to have at our disposal integrated metabolic and regulatory models for all organisms involved in cheese flavor as an essential step for the better understanding and, ultimately, steering of cheese flavor development. The models will be essential to determining what controls flux through key metabolic pathways. Also, questions such as how salt-in-the-moisture influences cheese flavor development are unlikely to be resolved without integrated metabolic and regulatory models.

Conclusions

Cheese flavor development is a complex and dynamic biochemical process. The factors that determine the flux through the critical metabolic pathways remain poorly understood and a systems biology approach is thus needed to advance current understanding of these processes. This will require advances in transcriptomics, proteomics and metabolomics as well as the development of integrated metabolic and regulatory models for key components of the cheese microbiota.

Prospects are great that before long major steps forward can be taken:

- Rapid improvements in next-generation sequencing (NGS) depth and quality offer unprecedented possibilities in the field covered by this review. Rapid screening by full-genome sequencing of random or targeted mutants, of strains obtained from experimental evolution experiments, or novel (environmental) isolates is now a real option. One major challenge, the identification of industrially exploitable information from the large and often noisy (flavor) phenotype and omics datasets, is tackled by the development of new tools [60**].
- The role that small RNAs [61] and lowly expressed genes might play in (regulating) flavor formation

pathways is fully unexplored in LAB. Also here, NGS will allow filling this serious knowledge gap. The 'noise' in the expression of lowly expressed genes and in genetic circuits are among the main driving forces for cell-to-cell variability, which results in cells within an isogenic bacterial population in one environment exhibiting dissimilar phenotypes [62–64]. Advanced (microscopic) technology is available to analyze the response of single cells in a culture to intrinsic perturbations or extrinsic (environmental) changes. Culture heterogeneity may be significant to industrial LAB phenotypes including flavor production and is becoming an important new area in both fundamental and application-oriented LAB research.

In summary, the combination of NGS, systems biology approaches and single-cell technology will ultimately reveal the complex physiology of starter cultures during cheese making and their intricate mutual interactions and communication with other microorganisms in cheese: who is doing what, where, when and with whom in the cheese matrix. Knowledge generated from these novel culture and single-cell approaches will yield technology (i.e. cultures, enzymes, and new processes) that provides rapid and consistent cheese flavor development.

Acknowledgments

Top Institute Food and Nutrition (JK) and Dairy Management, Inc., and the USDA (JS, JB) are gratefully acknowledged for financial support of part of this work.

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